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## Characterization of Two Non-Locus of Enterocyte Effacement-Encoded Type III-Translocated Effectors, NleC and NleD, in Attaching and Effacing Pathogens

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**Intestinal colonization by enteropathogenic and enterohemorrhagic *Escherichia coli* requires the locus of enterocyte effacement-encoded type III secretion system. We report that NleC and NleD are translocated into host cells via this system. Deletion mutants induced attaching and effacing lesions in vitro, while infection of calves or lambs showed that neither gene was required for colonization.**

Attaching and effacing strains of *Escherichia coli* (AEEC) comprise a group of gastrointestinal pathogens of humans and animals that induce distinctive attaching and effacing (A/E) lesions within the host intestinal mucosa (28). A/E lesions are characterized by intimate attachment of the bacteria to the host cell surface, the localized destruction of intestinal microvilli, and the rearrangement of host cytoskeletal proteins beneath the adherent bacteria (19). A/E lesion formation is mediated by the products of the locus of enterocyte effacement (LEE) pathogenicity island (PAI) (24). The LEE of enteropathogenic *E. coli* (EPEC) contains 41 open reading frames (11) of which approximately half encode a type III secretion system (TTSS), which directs the secretion of several LEE-encoded translocator (EspA, EspB, and EspD) and effector (EspG, EspH, Map, Tir, EspZ, and EspF) proteins. However, only Tir plays a direct role in A/E lesion formation (12, 13). The LEE PAI is well conserved among AEEC strains, including the closely related human pathogen enterohemorrhagic *E. coli* (EHEC) (30) and the animal pathogens rabbit-specific enteropathogenic *E. coli* (35) and *Citrobacter rodentium* (7).

Recently, several novel effector proteins that are not encoded within the LEE but are translocated into host cells by the LEE-encoded TTSS were described. Cycle inhibiting factor (designated Cif), although not required for A/E lesion formation, induces host cell cycle arrest and reorganization of the actin cytoskeleton (21). EspI/NleA (for non-LEE encoded) (Z6024) (15, 25) is also not required for A/E lesion formation but is essential for full virulence in the *C. rodentium* mouse model of infection and was found to be more frequently as-

sociated with EHEC strains isolated from symptomatic than from asymptomatic patients (26). EspJ (5) and TccP/EspF<sub>U</sub> (3, 14) are carried on prophage CP933U. In the murine model of infection, an *espJ* deletion mutant of *C. rodentium* showed altered colonization and clearance dynamics, although the protein was not required for A/E lesion formation (5). In contrast, TccP/EspF<sub>U</sub> is essential for A/E lesion formation by EHEC O157:H7 (3, 14) and disruption of the tight junctions (38). In EHEC O157:H7-infected cells, TccP functions as a linker between Tir and N-WASP and compensates functionally for the absence of the host adapter protein, Nck, which in EPEC-infected cells is recruited to the pedestal upon tyrosine phosphorylation of Tir (1, 2). EspG2, which is encoded on the EspC PAI, shares similarity with EspG (10) and was recently shown to interact with tubulin and trigger the dissociation of microtubules beneath adherent bacteria in a manner similar to that of EspG (23, 32).

Several other secreted proteins, encoded at different positions on the chromosome, have been identified through proteomic analysis of the secretome of *C. rodentium* and shown to be secreted by the LEE-encoded TTSS, although it is still unclear if these proteins constitute novel translocated effectors (8). These include NleB (Z4328), NleC (Z0986), NleD (Z0990), NleE (Z4329), and NleF (Z6020). Recently, the gene encoding NleD was identified during a signature-tagged mutagenesis screen as essential for full colonization of the bovine gut by EHEC O157:H7 strain EDL933 (9). In this study, we investigated whether NleD and another putative effector present in the same O-island, NleC, were translocated into host cells by the LEE-encoded TTSS and the contribution of *nleC* and *nleD* to virulence in several in vitro and in vivo models of infection.

**Sequence analysis of the putative type III-secreted effectors, NleC and NleD.** The respective EHEC O157:H7 homologs of NleC and NleD proteins, Z0986 and Z0990, show 95% and

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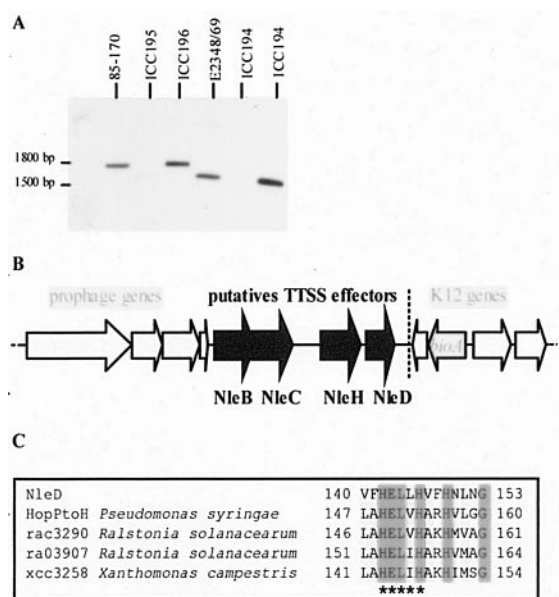


FIG. 1. The deletion of *nleC* (A) and *nleD* (not shown) was confirmed by Southern blot analysis. *NleD* and *NleC* are encoded at the 5' end of prophage CP-933K inserted in the *bio* operon located at 17.3 min in the K-12 chromosome. (B) The locus encoding *NleD* and *NleC* also encodes two other putative type III-secreted effectors, *NleB* and *NleH*. (C) Alignments of the zinc binding motifs (indicated by an asterisk) located in the C-terminal domains of *NleD* with type III-secreted effector proteins of plant pathogens.

84% amino acid similarity with their *C. rodentium* counterparts ([http://www.sanger.ac.uk/Projects/C\\_rodentium](http://www.sanger.ac.uk/Projects/C_rodentium)) and are encoded within prophage CP-933K in EHEC O157:H7 EDL933, which corresponds to O-island 36 (Fig. 1A). Z0986/*NleC* is predicted to be 330 amino acids in length with a molecular mass of 37 kDa; Z0990/*NleD* is predicted to be 232 amino acids with a molecular mass of 26 kDa. Sequences homologous to Z0986/*NleC* and Z0990/*NleD* are also present in the unfinished genome sequence of EPEC strain E2348/69 ([http://www.sanger.ac.uk/projects/Escherichia\\_Shigella](http://www.sanger.ac.uk/projects/Escherichia_Shigella)), sharing 100% and 99% similarity, respectively, with their counterparts in EHEC O157:

H7. Whereas Z0986/*NleC* shows no similarity to proteins of non-AEEC pathogens, Z0990/*NleD* exhibits 38% similarity with the type III effector HopPtoH from *Pseudomonas syringae* (31), 36% similarity with protein XCC3258 from *Xanthomonas campestris*, and 45% and 42% similarity with proteins RSc3290 and RS03907, respectively, from *Ralstonia solanacearum* strain GMI1000 (Fig. 1B). Although the function of these proteins is unknown, the shared similarity extends to a conserved zinc binding motif, which suggests that these proteins could have a metallopeptidase activity.

#### Secretion and translocation of *NleC* and *NleD* into host cells.

Recently, a system to study protein secretion and translocation was developed that utilizes translational fusions to TEM-1  $\beta$ -lactamase (4). TEM-1  $\beta$ -lactamase lacks a leader peptide sequence for export and can only be secreted if fused to a secreted protein. Likewise, TEM-1 translocation into host cells can only occur if TEM-1 is fused to a translocated protein. The presence of TEM-1 in infected eukaryotic cells can be measured directly by the addition of the fluorescent substrate CCF2-AM. Eukaryotic cells containing TEM-1 cleave CCF2-AM and appear blue under UV illumination, while cells lacking TEM-1 do not cleave CCF2-AM and appear green. To determine if Z0986/*NleC* and Z0990/*NleD* were translocated into host cells by the LEE-encoded TTSS, translational fusions with TEM-1 were constructed in plasmid pCX340 (Table 1). A 776-bp fragment containing the gene *nleD* was amplified by PCR using primers Z0990NdeIf and Z0990EcoRIr (Table 2) using genomic DNA from EPEC E2348/69 as a template. The PCR product was digested by NdeI and EcoRI and ligated into pCX340, generating plasmid pICC309 encoding an *NleD*-TEM-1 fusion. Plasmid pICC308 encoding an *NleC*-TEM-1 fusion was obtained in the same way after NdeI/EcoRI digestion and ligation of a 1,110-bp PCR product amplified with primers Z0986NdeIf and Z0986EcoRr (Table 2). Plasmids pICC308 and pICC309 were introduced into EPEC E2348/69 and an *escN* mutant of EPEC E2348/69, ICC192; the recombinant strains, grown in Dulbecco's modified Eagle medium (DMEM), were analyzed for protein secretion. Western blot analysis with antibodies to TEM-1 showed that the *NleC*-TEM-1 and *NleD*-TEM-1 fusion proteins were secreted into

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristic(s)	Reference or source
<b>Strains</b>		
EPEC E2348/69	Wild-type EPEC O127:H6	20
EHEC 85-170	Spontaneous <i>stx1</i> and <i>stx2</i> minus EHEC O157:H7	36
85-170 nal <sup>r</sup>	Spontaneous Nal <sup>r</sup> derivative of 85-170	33
ICC192	E2348/69 <i>escN</i> ::Kan	14
ICC195	85-170 <i>nleC</i> ::Kan (Nal <sup>r</sup> Kan <sup>r</sup> )	This study
ICC196	85-170 <i>nleD</i> ::Kan (Nal <sup>r</sup> Kan <sup>r</sup> )	This study
ICC193	E2348/69 <i>nleC</i> ::Kan	This study
ICC194	E2348/69 <i>nleD</i> ::Kan	This study
<b>Plasmids</b>		
pCX340	Cloning vector used to construct TEM-1 $\beta$ -lactamase fusions; Tet <sup>r</sup>	4
pKD4	<i>oriR101 blaM</i> ; Kan <sup>r</sup> cassette flanked by FRT sites	6
pKD46	<i>oriR101 repA101(ts) araBp-gam-bet-exo blaM</i>	6
pICC308	Derivative of pCX340 encoding an <i>NleC</i> -TEM-1 fusion	This study
pICC309	Derivative of pCX340 encoding an <i>NleD</i> -TEM-1 fusion	This study

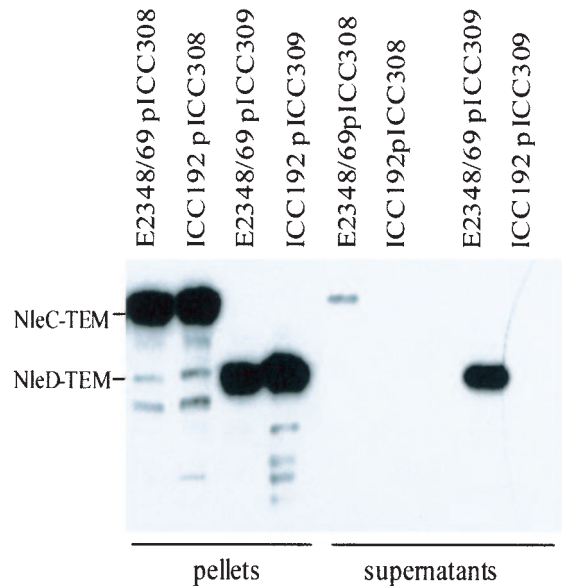
TABLE 2. Primers used in this study

Name	Primer sequence (5'-3')
Z0990 FRT f.....	ATGCGCCCTACGTCCCTCAACTTGGTATTACATCAGTCATCAAGGTCGAGTGTGTAGGCTGGAGCTGCTTCG
Z0990 FRT r.....	CTAAAGCAATGGATGCAGTCTTACCTGTTGGAATCCCAGACTCACTGTATCATATGAATATCCTCCTTAG
Z0986 FRT f.....	ATGAAATTCCTCTATTACAGTCCAACCTTCAACTTTTCCGCCCGGCGAGGTGTGTAGGCTGGAGCTGCTTCG
Z0986 FRT r.....	TCATTGCTGATGTGTGTTGTCCACATCCCCAAATGTAAAAATATAAGGCATATGAATATCCTCCTTAG
Z0990NdeI f.....	GGTATTCCATATGCGCCCTACGTCCCTCA
Z0990EcoI r.....	CGGAATTCCTCAAGCAATGGATGCAGTC
Z0986NdeI f.....	GGTATTCCATATGAAATTCCTCTATTAC
Z0986EcoRI r.....	CGGAATTCGCTTGCTGATTGTGTTTGTC

the culture supernatant by wild-type EPEC E2348/69 but not by ICC192, confirming that they are substrates of the LEE-encoded type III secretion system (Fig. 2A). The recombinant strains were then used to infect HeLa cell monolayers, and TEM-1 translocation assays were performed as described by Charpentier and Oswald (4). Wild-type and  $\Delta$ *escN*-EPEC E2348/69 strains were subcultured in LB supplemented with tetracycline and incubated for 16 h at 37°C. The cultures were diluted 1/100 in DMEM supplemented with 10% fetal calf serum and 2 mM glutamine at 37°C for 2.5 h (preactivation). HeLa cells grown on glass coverslips were infected with 1 ml of preactivated bacterial culture (multiplicity of infection, ~100:1) and incubated at 37°C in 5% CO<sub>2</sub>. After 30 min of infection, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, and the coverslips were incubated for an additional hour. Cell monolayers were washed three times with phosphate-buffered saline (PBS) and covered with 100  $\mu$ l of PBS plus 25  $\mu$ l of 6XCCF2/AM solution freshly prepared from the CCF2/AM Loading kit (CCF2/AM final concentration, 1  $\mu$ M; Invitrogen). The cells were incubated in darkness at room temperature for 2 h of culture. Cells were washed three times with PBS and observed under a Nikon Eclipse E600 fluorescence microscope with a UV-2A filter set (330- to 380-nm excitation). Images, captured using a Nikon DXM1200 digital camera, showed that both NleC-TEM-1 and NleD-TEM-1 fusion proteins were translocated into host cells by wild-type EPEC E2348/69 but not by ICC192, indicating that NleC and NleD constituted novel translocated effectors of the LEE-encoded TTSS (Fig. 2B).

**In vitro analysis of *nleC* and *nleD* mutants of EPEC and EHEC.** Although the LEE of EPEC encodes all the proteins required for A/E lesion formation, the prophage-carried effector TccP is additionally required for A/E lesion formation by EHEC O157:H7 (3, 14). Therefore, to examine the contribution of NleC and NleD to A/E lesion formation and adherence in vitro, we constructed *nleC* and *nleD* deletion mutants. Mutations in *nleC* and *nleD* genes for EPEC E2349/69 and EHEC 85-170 were constructed by using the PCR one-step  $\lambda$  red recombination system (6). Each mutation was obtained using a PCR substrate containing a kanamycin resistance gene flanked by the 50 bases from the 5' and 3' ends of the target gene. Plasmid pKD4 carrying the kanamycin resistance gene was used as the PCR template. The PCR product was electroporated into the recipient strains carrying the Red system expression plasmid pKD46, and mutants were selected on LB plates with kanamycin. Recombinant clones were cured of pKD46 plasmid by growth at the nonpermissive temperature (42°C), and mutation was confirmed by different PCRs using primers

A



B

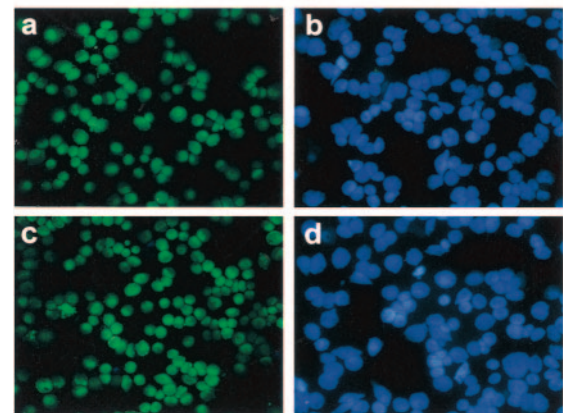


FIG. 2. NleC and NleD are secreted and translocated into eukaryotic cells in a LEE-encoded TTSS-dependent manner. (A) Wild-type EPEC E2348/69 strain and a strain mutated in *escN*, ICC192, expressing NleC-TEM or NleD-TEM fusions were grown in Dulbecco's modified Eagle medium; pellets and supernatants were probed by Western blot analysis for the presence of TEM fusion proteins. (B) The same strains, ICC192(pICC308) (a), EPEC E2348/69(pICC308) (b), ICC192(pICC309) (c), and E2348/69 (pICC309) (d), were used to infect HeLa cells; after infection, HeLa cells were washed and loaded with CCF2/AM. The presence of TEM fusions in HeLa cells is revealed by a blue fluorescence due to cleaving of CCF2/AM by the  $\beta$ -lactamase encoded by TEM, whereas uncleaved CCF2/AM substrate fluoresces green.



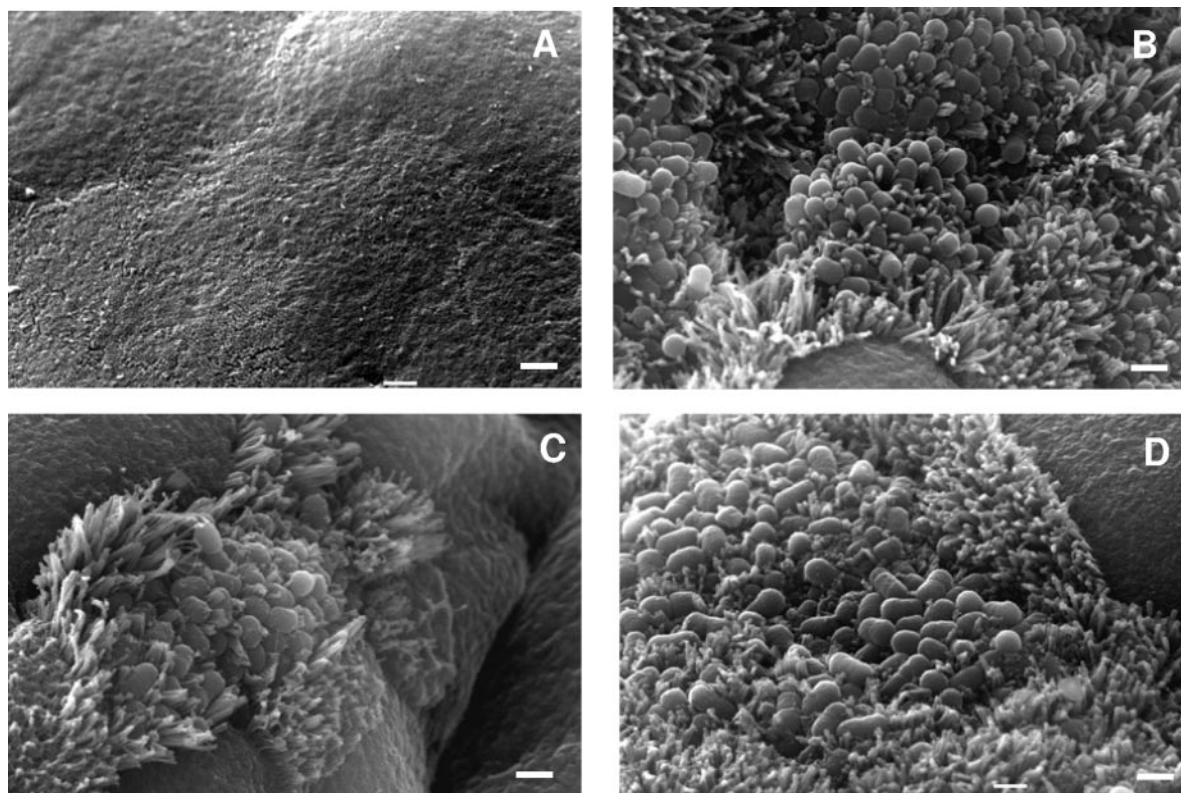


FIG. 3. NleC and NleD are not required for induction of A/E lesions. Scanning electron micrographs showing uninfected duodenal mucosa (A) and duodenal mucosa infected for 8 h with parental EPEC E2348/69 strain (B), *nleC* mutant strain ICC193 (C), and *nleD* mutant strain ICC194 (D). Size bar, 1  $\mu$ m.

flanking the targeted region and primers into the kanamycin resistance gene (not shown) and by Southern blotting (Fig. 1A) with HindIII/BglII-digested genomic DNA purified with a QBiogene GNOME DNA kit and a gene-specific PCR probe labeled with the ECL Direct Nucleic Acid labeling kit (Amersham Biosciences).

The ability of the *nleC* and *nleD* EPEC E2348/69 (ICC193 and ICC194, respectively) and EHEC 85-170 (ICC195 and ICC196, respectively) strains to induce A/E lesion in vitro (HEp-2 cells) and ex vivo (human intestinal in vitro organ cultures [IVOC]) was examined. The fluorescent actin staining test was performed on infected HEp-2 cells as described by Knutton et al. (18). For IVOC, tissue was obtained (after obtaining fully informed parental consent and local ethical committee approval) with grasp forceps during routine endoscopic investigation of intestinal disorders with a Fujinon EG/EC-41 pediatric endoscope. Proximal small intestinal mucosal biopsies from the fourth part of the duodenum, which appeared macroscopically normal, were taken for organ culture experiments. Light microscopy subsequently showed no histological abnormality. IVOC infections were performed as described previously (16). EPEC  $\Delta nleC$  (ICC193) and  $\Delta nleD$  (ICC194) were examined using tissue samples from three patients (aged 29, 118, and 142 months). In each experiment, a noninoculated sample (to exclude endogenous bacterial adhesion) and a positive control (IVOC with parent strain E2348/69 to exclude host factors) were included. Samples were fixed with 2.5% glutaraldehyde, postfixed in 1% aqueous osmium tetrox-

ide, and processed for viewing with a JEOL JSM 5300 scanning electron microscope (16).

No difference was seen in the ability of wild-type and *nleC* and *nleD* mutant strains of either EPEC or EHEC to induce actin polymerization on HEp-2 cells (data not shown). In addition, there was no difference in the pattern of adherence of wild-type and mutant EPEC strains to HEp-2 cells, which all exhibited localized adherence, or in the pattern of adherence of wild-type EHEC and mutant strains, which all exhibited diffuse adherence (data not shown). NleC and NleD are therefore not required for adherence to tissue culture cells or actin accumulation in EPEC and EHEC. Similarly, all strains tested were able to adhere to human intestinal tissue and induce A/E lesions (Fig. 3), confirming that NleC and NleD do not play a role in intimate attachment to human intestinal biopsy tissue in vitro.

**Colonization of lambs by *nleC* and *nleD* mutants of EHEC O157:H7.** As ruminants are considered an important reservoir of EHEC, we therefore determined the contribution of NleC and NleD to colonization of lambs and calves. Fifteen 6-week-old cross-bred lambs were randomly divided into three equal groups, supplied with food and water ad libitum, and confirmed to be free of EHEC O157 by enrichment and O157 immunomagnetic separation. All lambs were housed in bio-secure containment level 2 accommodations. Each group was housed in a separate room with its own air handling. Animals were visited only by experienced staff who changed clothing between each group. Five lambs were each dosed orally with

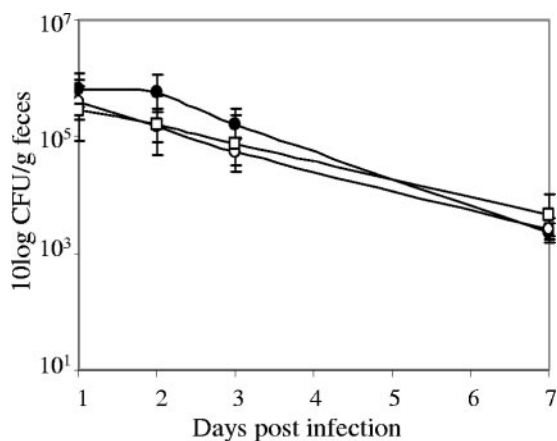


FIG. 4. The translocated effector proteins NleC and NleD are not required for persistence of EHEC O157:H7 in lambs. Colonization is indicated by viable bacterial counts from fecal samples taken at different time points postinfection. Lambs were infected with ca.  $10^9$  CFU of the EHEC O157:H7 85-170 parental strain (closed circles), *nleC* mutant (open squares), or *nleD* mutant (open circles). Error bars show standard deviations.

$10^9$  CFU of either 85-170 *Nal*<sup>r</sup>, 85-170 $\Delta$ *nleC*::*Kan*<sup>r</sup>, or 85-170 $\Delta$ *nleD*::*Kan*<sup>r</sup> resuspended in 10 ml of PBS (pH 7.4). Approximately 24 h after dosing and as required thereafter for up to 27 days, rectal fecal samples from each lamb were collected for direct plating onto sorbitol-MacConkey (SMAC; Oxoid, Basingstoke, United Kingdom) plates supplemented with either 15- $\mu$ g/ml nalidixic acid or 25- $\mu$ g/ml kanamycin (Sigma). Samples which were negative by direct plating were enriched in buffered peptone water for 6 h at 37°C and then plated onto SMAC supplemented with the appropriate antibiotic. Representative colonies were confirmed to be *E. coli* O157 by latex agglutination (Oxoid, Basingstoke, United Kingdom). The ability of the test strains to establish and persist in lambs was investigated by monitoring viable bacteria recovered in stools collected per rectum. In this model, wild-type EHEC 85-170 produced a typical shedding pattern, persisting in high numbers in the early stages of infection and then declining until undetectable by day 10 postinfection (Fig. 4). Wild-type EHEC 85-170 then remained undetectable by direct plating or with enrichment for the remainder of the study (27 days in total; data not shown). In contrast, while ICC195 and ICC196 showed levels of colonization that were similar those of to wild-type EHEC 85-170 in the early stages of infection, one animal continued to shed ICC196 (as detected by enrichment) until 14 days postinfection (data not shown).

**Coinfection of calves with EHEC O157:H7 wild-type and *nleD* mutant.** Recently, an EHEC O157:H7 strain EDL933 mutant harboring a transposon insertion in *nleD* was identified by signature-tagged mutagenesis as being attenuated following inoculation of 10- to 14-day-old calves (9). Accordingly, we tested the defined *nleD* mutant in coinfections. Conventional Friesian bull calves aged 10 to 14 days were housed in high-containment accommodations in tanks on tenderfoot mats and fed on milk replacer twice daily with free access to water. Prior to infection, calves were confirmed to be culture negative for EHEC and *Salmonella* by direct plating of rectal swabs on sorbitol-MacConkey agar containing 2.5- $\mu$ g/ml potassium tel-

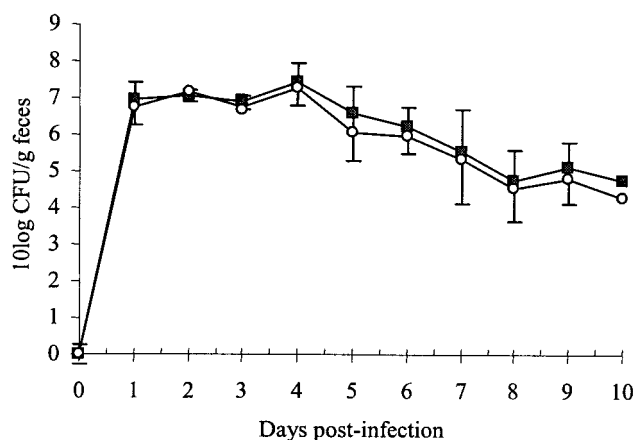


FIG. 5. Course of fecal excretion of EHEC O157:H7 strains 85-170 *Nal*<sup>r</sup> and 85-170 *Nal*<sup>r</sup> *nleD*::*Kan*<sup>r</sup>, following coinfection of three ca. 14-day-old calves. Mean  $\log_{10}$ -transformed shedding data ( $\pm$  the standard error of the mean) for the wild-type (closed squares) and mutant (open circles) strains is shown.

lurite (T-SMAC) or Brilliant Green agar (Oxoid, Basingstoke, United Kingdom), respectively. Presumptive EHEC were screened for *stx1* and *stx2* genes as previously described (33) and animals excreting *stx*-positive *E. coli* or *Salmonella* were excluded. All calves were obtained from the same farm and received colostrum from their respective dams for the first 24 to 48 h. Thereafter, no further colostrum was given. Total serum-immunoglobulin levels were measured at 1 or 2 days of age as a measure of colostrum intake by zinc sulfate turbidity assay. Only calves with a zinc sulfate turbidity measurement of  $>10$  were used.

Bacterial strains for inoculation were amplified in brain heart infusion broth for 18 h at 37°C and adjusted to the same optical density. Three calves were orally challenged with ca.  $1 \times 10^{10}$  CFU of a 1:1 mixture of 85-170 *Nal*<sup>r</sup> and the defined 85-170 *Nal*<sup>r</sup> *nleD*::*Kan*<sup>r</sup> mutant. Fecal samples were collected once daily for 10 days by rectal palpation; calves remained asymptomatic throughout. The amount of viable EHEC per gram of feces was enumerated by plating triplicate 10-fold serial dilutions onto T-SMAC containing 2.5- $\mu$ g/ml potassium tellurite and 20- $\mu$ g/ml nalidixic acid (T-SMAC-Nal) and T-SMAC-Nal containing 50- $\mu$ g/ml kanamycin (T-SMAC-Nal-Kan). The number of wild-type bacteria was calculated by subtracting the viable count on T-SMAC-Nal-Kan from that obtained using T-SMAC-Nal. Recovery of the mutant strain in the feces was confirmed by PCR from selected colonies on T-SMAC-Nal-Kan plates using the primers 5'-ACAGAG ACAAATGTCTTATATGA-3' and 5'-CAGTCATAGCCG AATAGCCT-3'. The fecal shedding data were statistically analyzed for the effect of mutation by means of an F-test, with the data taken as repeated measurements (Proc Mixed; Statistical Analysis System, SAS Institute, Cary, N.C.). *P* values of  $<0.05$  were taken to be significant. The  $\log_{10}$ -transformed fecal excretion data show that the magnitude and duration excretion of the *nleD* mutant were very similar to those of the wild type; no statistically significant reduction in shedding of the mutant could be detected at any time postinoculation (Fig. 5).

The LEE-encoded TTSS of AECC is essential for virulence

and translocates at least 10 proteins from the bacterial cytoplasm into the host cell cytosol (13). Some of the translocated effectors have been shown to be essential for colonization and disease in various animal models of infection, including EspB (8, 29) and EspD (8, 27), which together with EspA form the EPEC/EHEC translocon required for the delivery of other effector proteins into host cells (12). Tir (17, 22, 33), which is essential for intimin binding and intimate attachment of the bacteria to the host cell surface, and EspI/NleA are also essential for virulence in several models of infection (15, 25); however, other translocated effector proteins appear to make little contribution to pathogenesis in animal infection models (8, 25). Therefore, the contribution of various translocated effectors to colonization and disease varies considerably.

Until recently, the only effector proteins known to be translocated by the LEE-encoded TTSS were also encoded within the LEE (12). However, several novel effector proteins have now been identified that are not encoded by LEE, yet they are secreted and translocated into cells by the LEE-encoded TTSS (13). Two of these putative non-LEE-encoded effectors, NleC and NleD, were identified in *C. rodentium* by proteomic analysis of secreted proteins (8). The genes encoding NleC and NleD are present in other AECC strains, including EPEC and EHEC, and are located in O-island 36 of the EHEC O157:H7 EDL933 genome. While NleC has no similarity with proteins from non-AECC strains, NleD shares some similarity with the TTSS effector, HopPtoH, from the plant pathogen *P. syringae* (31). Although the contribution of HopPtoH to the virulence of *P. syringae* is unknown, both proteins share a common zinc binding motif found in the neurotoxin of *C. botulinum* which may be important for their function.

In this study, we demonstrated that NleC and NleD are true translocated effectors of the LEE-encoded TTSS. An intact LEE was required for the secretion of NleC-TEM-1 and NleD-TEM-1 fusion proteins and for the translocation of the fusion proteins into host cells. The observations that NleC and NleD could enter cells suggested that these proteins may play a direct role in host-pathogen interactions. To determine the contribution of the proteins to virulence, we constructed *nleC* and *nleD* deletion mutants in strains of EPEC and EHEC and tested the mutants in several in vitro and animal models of infection. The results showed that neither NleC nor NleD played a role in A/E lesion formation, adherence to human intestinal tissue, adherence to tissue culture cells, or carriage and virulence in lambs or calves. Importantly, the calf model is capable of detecting attenuation, as we have previously reported that mutation of EHEC O157:H7 *escN*, *tir*, or *z2200* and EHEC O5/O111 *efal* causes a highly significant reduction in fecal excretion of the respective mutants by 7 to 10 days post-inoculation (9, 33, 34, 37).

As EDL933 *nleD* mutant was identified by signature-tagged mutagenesis as being attenuated following inoculation of 10- to 14-day-old calves (9), we confirmed in separate coinfection studies that the EDL933 *NaI<sup>r</sup> nleD::miniTn5Km2* mutant (11A3) was significantly attenuated relative to the parent strain in three calves ( $P < 0.01$  at 6 days postinoculation; data not shown). While this is consistent with the attenuation observed in the initial signature-tagged mutagenesis screen (9), the data with the defined 85-170 *nleD::Kan<sup>r</sup>* mutant in calves and lambs imply that the attenuation of the EDL933 transposon mutant

may be the result of a second-site defect or strain-specific effect. At this stage, the precise role of NleC and NleD in the pathogenesis of infections with AECC is unclear. Although in this study we were unable to find a phenotype for NleC or NleD, the fact that they are translocated into host cells suggests that the proteins have the potential to influence host-pathogen interactions. Our data suggest that as new putative effector proteins emerge, the challenge will be to pinpoint their role in infection, which may vary in different hosts and with different bacterial isolates. This problem may be compounded by the presence of one or several homologs in the one pathogen, as seen for EspF and EspG, which could offer more functional redundancy among the translocated effectors.

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